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Noninvasive Dopamine Determination by Reversed Phase HPLC in the Medium of Free-Floating Roller Tube Cultures of Rat Fetal Ventral Mesencephalon: A Tool to Assess Dopaminergic Tissue Prior to Grafting

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ABSTRACT: The low availability of dopamine containing neurons for grafting in Parkinson's disease is a general problem. Free-floating roller tube (FFRT) cultures allow storage of fetal mesencephalic tissue prior to transplantation. Preoperative functional testing permits to select an optimized set of individual cultures for transplantation. Rat fetal ventral mesencephali (E13) were dissected out and divided into four equally sized pieces each and individually prepared as FFRT cultures. After 4, 8, 12, and 16 days in vitro (DIV) the medium of each culture was collected during routine medium change and immediately stabilized. Dopamine was extracted and probes were determined with reversed phase HPLC using electrochemical detection. After 16 DIV cultures were fixed and cell counts performed in tyrosine hydroxylase (TH)-immunostained serial sections. The mean dopamine content ± SEM in culture conditioned media was at 4 DIV: 21 ± 2 pg, n = 38; at 8 DIV: 37 ± 4 pg, n = 40; at 12 DIV: 52 ± 7 pg, n = 38; and at 16 DIV: 39 ± 5 pg, n = 38. In all cultures devoid of dopamine after 4 and 8 DIV (12.5%) levels remained below detectability at 12 and 16 DIV. Cultures derived from the rostral mesencephalon showed significantly higher dopamine values than those from the caudal mesencephalon at 12 DIV. The mean number of TH-immunoreactive (-ir) cells/culture ± SEM after 16 DIV was 556 ± 51, n = 40. The correlation between TH-ir cell number (CN) and dopamine content of rostrally derived cultures at 16 DIV was: CN = 7.4 (dopamine [pg]) + 248; R = 0.75; n = 19; $p < 0.001$. No dopamine was present in cultures without TH-ir cells. These results demonstrate that sequential noninvasive screening of dopamine in single cultures is feasible and that the dopamine content is correlated to the number of surviving TH-ir cells. This permits to select cultures rich in dopaminergic neurons for transplantation.

KEY WORDS: Functional assessment, Tissue culture, Substantia nigra, Catecholamine, Fetal tissue.

INTRODUCTION

In recent years neural transplantation has become a promising tool beyond animal experimentation and has proven its clinical applicability in severely disabled patients with Parkinson's disease. Beneficial effects of fetal neural grafts have been observed in several cases [11–13,25,26,39], although the degree of clinical recovery has remained limited so far. The number of surviving dopaminergic neurons is one crucial factor for successful grafting, as previously demonstrated in various animal studies [5,33]. The treatment of the tissue with neurotrophic and neuroprotective agents before, during, and after transplantation has been proposed to increase the number and function of surviving dopaminergic neurons (for review [24]). Unfortunately, no method has been available to monitor noninvasively the consequences of growth factor supplementation prior to transplantation in a clinical setup. Moreover, tyrosine hydroxylase-immunoreactive (TH-ir) cell number has been shown to vary considerably within tissue derived from the same fetal mesencephalon [36] and to depend on the developmental stage and localization. Selective pooling of tissue with a high dopamine production would increase the ratio of dopaminergic to nondopaminergic neurons and thereby improve specificity of grafts.

The number of implanted cells has been routinely determined in suspension grafts by assessing cell density and viability by the trypan-blue exclusion test or by the acridine orange/ethidium bromide method [4,7]. As this procedure is unspecific in regard to cell type, neither a general nor a regional quantification of dopaminergic cell number can be given. Attempts have been made to confirm the presence of dopaminergic neurons in the graft by measuring homovanillic acid (HVA) in the medium of tissue strands prior to transplantation [10]. The direct determi-

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nation of dopamine has been attempted, but low extracellular dopamine concentration due to effective reuptake mechanisms and a rapid decay under culture conditions [half life time of dopamine in our medium = 19 minutes at pH 7.4 at room temperature (data not shown)] complicate a reliable in vitro assessment. The method presented here is based on dopamine determination by reversed phase HPLC in the medium of free-floating roller tube (FFRT) cultures, a culture system designed for in vitro maintenance of neural tissues prior to transplantation [35,36]. By immediate stabilization of the dopamine after medium change and its extraction by aluminiumoxide binding repetitive, noninvasive dopamine measurements can be carried out for individual cultures in a routine manner. The dopamine content as measured by HPLC analysis can be reliably correlated to the number of TH-ir neurons in culture. This allows the selection of cultures rich in TH-ir neurons for transplantation and may help to identify useful modifications in the in vitro handling of the cultures and to test the efficacy of preoperative tissue treatment with neurotrophic and neuroprotective agents.

METHOD

Identification and Tissue Culture Preparation

Fetal ventral mesencephalon of embryonic day 13 (E13) rat fetuses was dissected out according to Dunnett and Björklund [8]. Ventral mesencephali were divided by a rostrocaudal cut and again by a coronal cut into four equally sized pieces that were individually transferred into conical plastic tubes (Falcon No. 2095) so that one tube contained one culture free-floating in 1 ml of medium. Medium (100 ml) consisted of 55 ml Dulbecco's modified Eagle medium (DMEM) (Gibco No. 041-02320 M), 32.5 ml Hanks balanced salts solution (HBSS) (Gibco No. 041-04020 M), 1.5 ml glucose 20%, 10 ml fetal calf serum (FCS) (Gibco No. 013-06290), and 1 ml of 23.8% HEPES solution. One milliliter triple-antibiotic (Gibco No 061-05240 D) per 100 ml medium was added during the first 4 days in vitro (DIV). The tubes were placed in a roller drum (60 revolutions per hour) in an incubator (37°C) with 5% CO_2. The drum was tilted 5°. New empty tubes were saturated in 5% CO_2 in the incubator for 48 h prior to use to improve pH stability during the first day in vitro [17]. Medium change took place regularly at 4-day intervals. A detailed description of the roller tube method and the modifications used in the present study has been given in previous publications [15,16,36].

Dopamine Determination by Reversed Phase HPLC

At 4, 8, 12, and 16 DIV the medium (1 ml) of each culture was collected during routine medium change in 1.5 ml Eppendorf tubes that were previously filled with 0.22 mg metabisulfite in 50 μl orthophosphoric acid (7.5%) to allow immediate stabilization of dopamine. After centrifugation at 3000 × g through ultrafilters (Ultracent®-10, Bio-Rad Laboratories, CA) to eliminate proteins and other macromolecular constituents from the probes, dopamine was extracted by aluminium adsorption and eluted in 120 μl elution buffer using an analytical kit (Chromosystems, No. 5000). Samples were injected by means of an autosampler (ASI-5, Talbot, Switzerland) using a 100 μl injection loop. Separation was achieved by isocratic elution using a reverse-phase C18 column (Grom-Sil 80, ODS 2, 5 μ, 250 × 4.6 mm, Grom, Germany) in a commercially available mobile phase (Chromosystems, No 5001). The flow rate was adjusted to 1 ml/min (HPLC-Pump, S-1020, Sykam, Switzerland) resulting in a working pressure of 150 bar and leading to an elution time of 24 min for dopamine. Quantification was achieved by electrochemical detection with a dual electrode analytic cell (ESA Mod. 5011) with the oxidative potential set at 400 mV. The potential of the guard cell (ESA Mod. 5020) was set at 500 mV to eliminate electroactive impurities in the mobile phase prior to injection.

Tyrosine Hydroxylase Immunohistochemistry

Cultures were individually fixed in 4% paraformaldehyde and 0.16% picric acid in 0.1 M phosphate buffer (PB) at 4°C for 1 h and then equilibrated in PB with 20% sucrose at 4°C for 24 h. Single cultures were cut in 20 μm steps on a freezing microtome (2800 Frigocut N, Reichert–Jung) and adhered to a gelatine/Chrome-alum precoated glass carrier. For the immunohistochemical staining, the sections were first incubated in 0.3% Triton X-100/phosphate-buffered saline (PBS) solution for 30 min, then in 1.5% normal goat serum (Vectastain-kit PK 4001, Vector Laboratories Burlingame, CA) for 20 min, and finally exposed to 0.048 μg/ml (1:500) anti-TH-antibody (Pel-Freeze Bio, P40101-0 RBT TH) at 4°C for 12 h. Thereafter the sections were incubated with a biotinylated antirabbit antibody (Vectastain-kit PK 4001) at a concentration of 1:200 in PBS for 30 min. Endogenous peroxidase was inhibited by 3.3% H_2O_2/10% methanol in PBS. Specifically bound antibody was detected following incubation with avidin and a biotinylated horseradish peroxidase complex (Vector Lab. Burlingame, CA) for 45 min and visualized with a metal-enhanced 3,3′-Diaminobenzidine (DAB) substrate kit (Pierce, No. 34065) for 10 min at room temperature. Between all incubation steps sections were washed in 0.1 M PBS.

Histological Assessment

Based on a uniform random selection procedure 358 sections of the 40 cultures (mean ± SEM: 9 ± 1 sections/culture) were chosen for further quantification. All TH-ir cells as defined by an intense TH staining, a well-preserved cell structure and the presence of a cell nucleus were eligible for quantification and were counted in a blinded manner by microscopical observation. The resulting cell number was corrected for slice thickness and nucleus size according to Abercrombie [1].

Statistical Analysis

A commercially available statistical software package (Statistica 5.0, Statsoft, OK) was used for statistical analysis. The number of TH-ir cells and the dopamine content at the different DIVs were compared by the nonparametric Mann–Whitney U-test. The relationship between cell number and dopamine content as well as between dopamine measurements at different DIV was nonparametrically and parametrically tested by the Spearman and the Pearson correlation and quantified using a linear regression model. All results in text and in graphs are given as the mean ± SEM.

RESULTS

General Description of FFRT Cultures and Their Cytoarchitecture

All 40 FFRT cultures could be successfully maintained in vitro for 16 DIV (planned experimental period) and stained for TH. Culture size as given by the maximum diameter of the spherical cultures after 16 DIV was 1.06 ± 0.02 mm (min. 0.80 mm, max. 1.44 mm, n = 40). TH-ir cells were present throughout the culture volume, although in many cases a gradient in differentiation, with rather immature cells in the center of the culture and well differentiated dopaminergic neurons at the periphery, could

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be detected (Fig. 1A). Often, a dense network of TH-ir fibres was present in the periphery of the cultures as described earlier for fetal mesencephalic FFRT cultures of human origin [36]. TH-ir neurons were of oval shape with one to four primary neurites emerging from the apical and basal portions of the soma resulting in a bipolar orientation of the neurites (Fig. 1B). Only few spines and varicosities were present on TH-ir neurites.

HPLC Measurements of Dopamine Content in the Medium of Cultures at Different Times In Vitro

The mean dopamine content ± SEM in the medium of FFRT cultures for the different DIVs was: 4 DIV 21 ± 2 pg, $n = 38$; 8 DIV 37 ± 4 pg, $n = 40$; 12 DIV 52 ± 7 pg, $n = 38$; 16 DIV 39 ± 5 pg, $n = 38$. Interestingly, cultures derived from the rostral part of the mesencephalon showed slightly increased dopamine levels at 8 DIV and significantly increased levels at 12 DIV ($p < 0.05$) compared to cultures derived from the caudal part, whereas no significant difference was present between rostrally and caudally derived cultures at 4 DIV and 16 DIV (Fig. 2). By separately analyzing cultures derived from the left and the right side of the same rostrocaudal localization nearly identical dopamine levels for the left and the right side were found, which supports the validity of the rostrocaudal differences in dopamine content over time (Fig. 2). All cultures with no detectable dopamine in the medium at 4 DIV ($n = 3$) remained without dopamine during the whole culture period, but six cultures with measurable dopamine (15 ± 3 pg, $n = 6$) at 4 DIV fell to a dopamine level of zero by 16 DIV. At 12 and 16 DIV between 78 and 81% of dopamine was present in the medium of 50% of the cultures assessed.

The dopamine levels of single cultures at different time points were significantly correlated (Figs. 3A–C). The best correlation was detected between values at 12 and 16 DIV (Fig. 3C): $r = 0.83$, $n = 38$, $p < 0.001$ (Pearson); $r = 0.84$, $n = 38$, $p < 0.001$ (Spearman). The correlations between the values at 4 DIV and those at 8 DIV (Fig. 3A) and between 8 DIV and 12 DIV (Fig. 3B) were lower: 4 DIV and 8 DIV: $r = 0.69$, $n = 38$, $p < 0.001$ (Pearson); $r = 0.64$, $n = 38$, $p < 0.001$ (Spearman); 8 DIV and 12 DIV $r = 0.61$, $n = 40$, $p < 0.001$ (Pearson); $r = 0.67$, $n = 40$, $p < 0.001$ (Spearman).

Histological Assessment

After 16 DIV immunohistochemical staining for TH was carried out. The cell counting revealed a mean number of TH-ir cells/culture ± SEM of 556 ± 51, $n = 40$, range 0–1333. No significant difference could be detected between cultures derived from the rostral part (598 ± 71, $n = 20$, range 0–1050) and the caudal part (534 ± 76, $n = 20$ range 0–1333) of the mesencephalon. The frequency histogram shows that most cultures comprised of 600 to 800 TH-ir cells (Fig. 4A). The distribution pattern for the number of TH-ir cells was very similar to that for the dopamine content at 16 DIV (Fig. 4B) with a maximum frequency between 60 and 80 pg. The total number of TH-ir cells derived from one mesencephalon was 2265 ± 265.

Correlation Between Dopamine Content and the Number of TH-ir Cells

Dopamine contents at the various time points were correlated to the number of TH-ir cells of the same cultures. The best correlations could be achieved between the TH-ir cell number of cultures derived from the rostral part of the fetal mesencephalon and the dopamine content at 12 DIV (Fig. 5A: $r = 0.77$, $n = 19$, $p < 0.001$ (Pearson); $r = 0.72$, $n = 19$, $p < 0.001$ (Spearman))

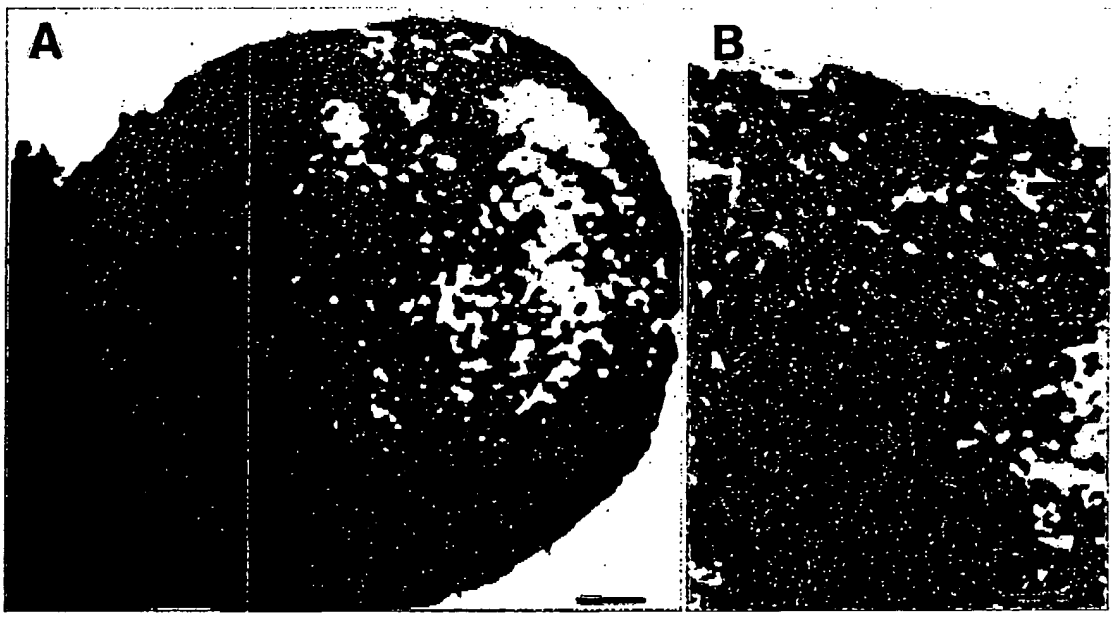

FIG. 1. (A) Overview of a 20 μm frozen section of a free-floating roller tube culture 16 DIV immunostained for TH. The cytoarchitecture reveals that TH-ir neurons are preferentially located around the perimeter of the culture (calibration bar: 100 μm; objective 10×, bright-field illumination). Arrow points to the region shown in B at higher magnification. (B) TH-ir neurons of the culture shown in A. Intensely stained somata with few neurites emerging from apical and basal portion can be readily recognized (calibration bar: 25 μm; objective 40×, bright-field illumination). This culture had a dopamine level in the medium of 44 pg at 16 DIV.

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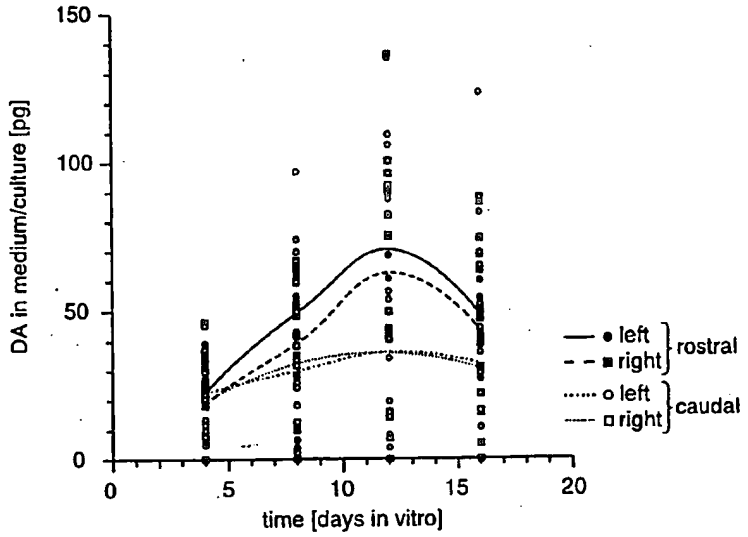

FIG. 2. Dopamine levels of individual FFRT-cultures in the medium after 4, 8, 12, and 16 DIV. Cultures derived from the left and the right side of the rostral and from the left and the right side of the caudal mesencephalon are shown separately. Left and right side derived cultures of the same rostrocaudal level demonstrate very similar dopamine levels while clear differences could be detected between rostrally and caudally derived cultures at 12 DIV ($p < 0.05$). Data are fitted by distance weighted least square method.

and at 16 DIV (Fig. 5B: $r = 0.75$, $n = 19$, $p < 0.001$ (Pearson); $r = 0.70$, $n = 19$, $p = 0.001$ (Spearman)). The correlations between the TH-ir cell number of cultures derived from the caudal part of the fetal mesencephalon and the dopamine content were as follows: 12 DIV: $r = 0.52$, $n = 19$, $p < 0.05$ (Pearson); $r = 0.59$, $n = 19$, $p < 0.01$ (Spearman); 16 DIV: $r = 0.40$, $n = 19$, $p = 0.09$ (Pearson); $rz = 0.47$, $n = 19$, $p < 0.05$ (Spearman). All cultures without TH-ir cells showed no detectable dopamine after 4, 8, 12, and 16 DIV. However, five cultures without dopamine contained a low number of TH-ir neurons (229 ± 72 cells). This suggests that TH immunoreactivity is a necessary but not sufficient condition for detectable dopamine levels in the medium of these cultures. The correlation between TH-ir cell number and dopamine content and the function of the linear fit are given for rostrally derived cultures (Fig. 5A,B) and pooled for rostrally and distally derived cultures (Fig. 5C,D) at 12 DIV and 16 DIV, respectively. The y-axis intercept is between 195 and 336 TH-ir cells. This corresponds to the mean cell number

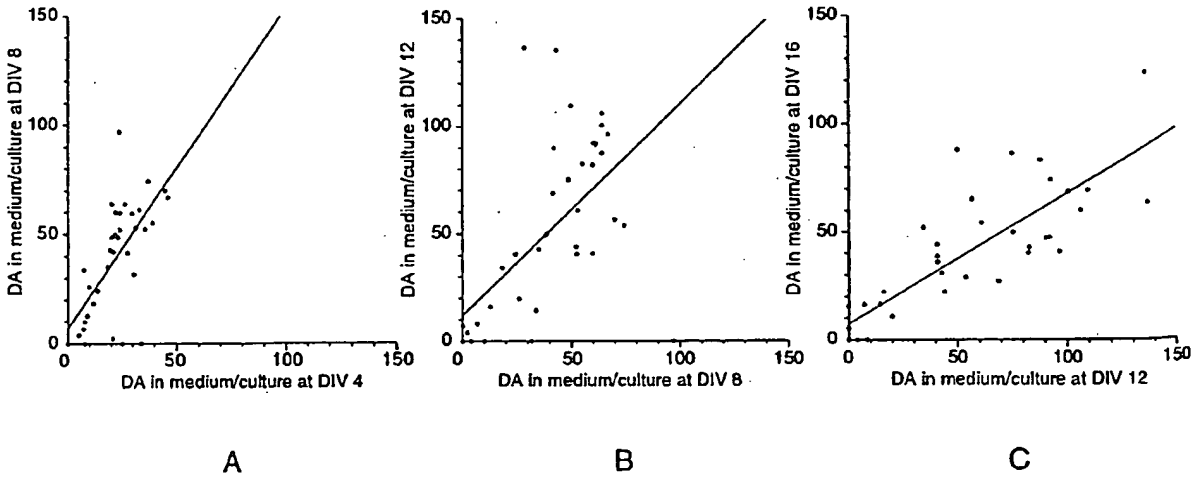

FIG. 3. (A) Correlation between the dopamine levels measured at 4 DIV and 8 DIV. (B) Correlation between dopamine levels measured at 8 DIV and 12 DIV. (C) Correlation between dopamine levels measured at 12 DIV and 16 DIV. Several data points in A and B are localized on the x-axis of the graphs with detectable amounts of dopamine at the first measurement but without dopamine 4 days later. Most of these cultures remained at zero levels in the following assessments.

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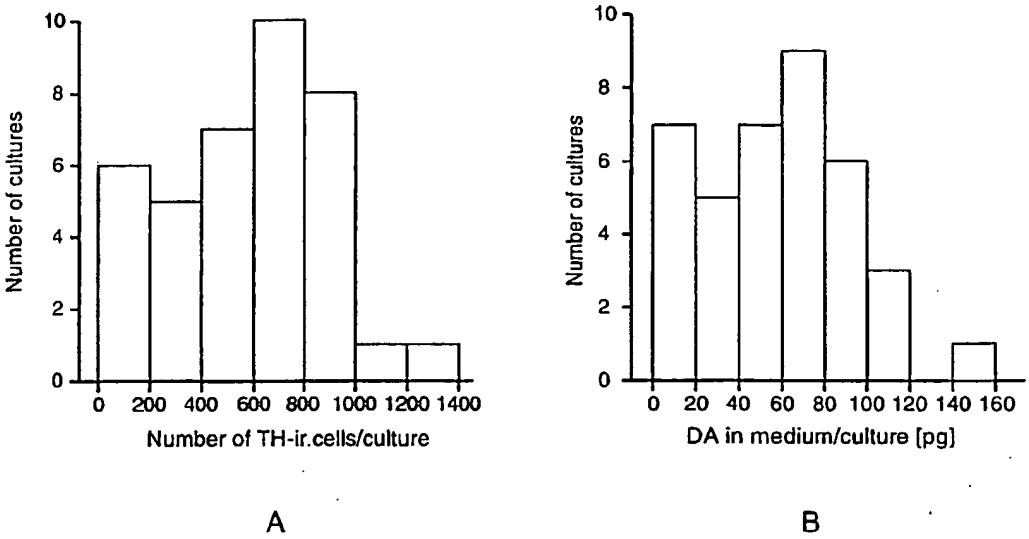

FIG. 4. (A) Frequency histogram of TH-ir cell numbers in each culture at 16 DIV. (B) Frequency histogram of dopamine levels of each culture at 16 DIV. A very similar overall distribution is visible in the two histograms. The frequency of cultures with less than 200 TH-ir cells was 15%, whereas that of cultures with less than 20 pg dopamine in the medium was 17.5%.

in the five cultures without detectable dopamine but histologically confirmed TH-ir cells. Thus, about 250–300 TH-ir cells are required for detectable dopamine levels in the medium.

DISCUSSION

This study shows the feasibility of repeated, noninvasive dopamine measurements in conditioned medium of rat fetal mesencephalic FFRT-cultures by reversed phase HPLC with electrochemical detection. Significant correlations between dopamine content of culture medium at different days in vitro and between dopamine content and TH-ir cell number 16 DIV demonstrate the validity of this method. Thus, a prediction of the dopaminergic cell number in individual cultures and the selective pooling of tissue rich in TH-ir neurons can be achieved. The transiently increased dopamine content at 12 DIV in cultures derived from the rostral mesencephalon as compared to that of caudally derived cultures may reflect time restricted developmental events in the maturation of dopaminergic neurons or precursors. This system may be suitable for quantitative monitoring of the effects of a wide range of in vitro manipulations to improve the number and function of dopaminergic neurons prior to transplantation.

The Use of FFRT Cultures in Neural Transplantation

Transplantation of FFRT cultures of rat and human origin [36] has several advantages as compared to that of classic roller tube cultures [29,31], fresh suspension grafts [3,4], or fresh solid grafts [14]. An increased donor age window for successful grafting has been demonstrated in solid grafts of rat and human origin as compared to suspension grafts [14,30]. In FFRT cultures no tissue dissociation and only minimal disruption of neuritic contacts is required for preparation and for implantation of the cultures in the host brain, whereas organotypic slice cultures have to be disrupted to be loaded into the implantation needle [31]. The delay time between explantation and transplantation allows pooling tissue of several embryos, sterility testing, preoperative treatment of the cultures to improve cell number and function [34], and noninvasive functional testing to assess dopamine production.

Survival rates of dopaminergic neurons are critical for successful grafting [5,33]. In this study the number of TH-ir neurons in FFRT cultures derived from one E13 rat mesencephalon was 2265 ± 265 after 16 DIV. Fawcett and co-workers reported that the total number of TH-ir neurons per rat mesencephalon (CFHB rat) was 37,482 at E14 [9]. Based on this study a survival rate of approximately 6% would result after 16 DIV. However, differences in cell numbers among different strains of rats, a difference in gestational ages (E13 rats used in the present study vs. E14 rats used by Fawcett and co-workers [9]) and differences in the excision and staining technique might attribute to miscellaneous estimations of the survival rate. Therefore, the evaluation of the number of TH-ir neurons in fresh explants would present a more accurate estimation of the survival rate and is an important issue for future comparative investigations. Brundin and coworkers [5,6] reported that at least 120 surviving TH-ir neurons are needed to induce behavioral changes in adult 6-OHDA-lesioned rats after intrastriatal grafting. In a preliminary experiment we have grafted rat fetal mesencephalic FFRT cultures, which were first maintained in vitro for 7 days in rats with unilateral 6-OHDA lesions. The observed reduction in amphetamine-induced rotation behavior corresponded to the survival of 400–800 TH-ir neurons per grafted rat fetal mesencephalon 12 weeks posttransplantation (Meyer and Spenger, unpublished data).

Dopamine Levels in Fetal Mesencephalic Tissue

Determination of dopamine using HPLC with electrochemical detection has been carried out in tissue derived from the rat [19,20,40] and human fetal mesencephalon [22,34,37,40]. In conditioned medium of dissociated rat and human fetal mesencephalic cultures dopamine determination has been carried out by Zhou and co-workers [40]. The dopamine levels reported by Zhou and co-workers [40] were about 18 pmol/ml (2700 pg/ml) medium in cultures of human fetal mesencephalon at 13 DIV and substantially lower (no precise data published) in condi-

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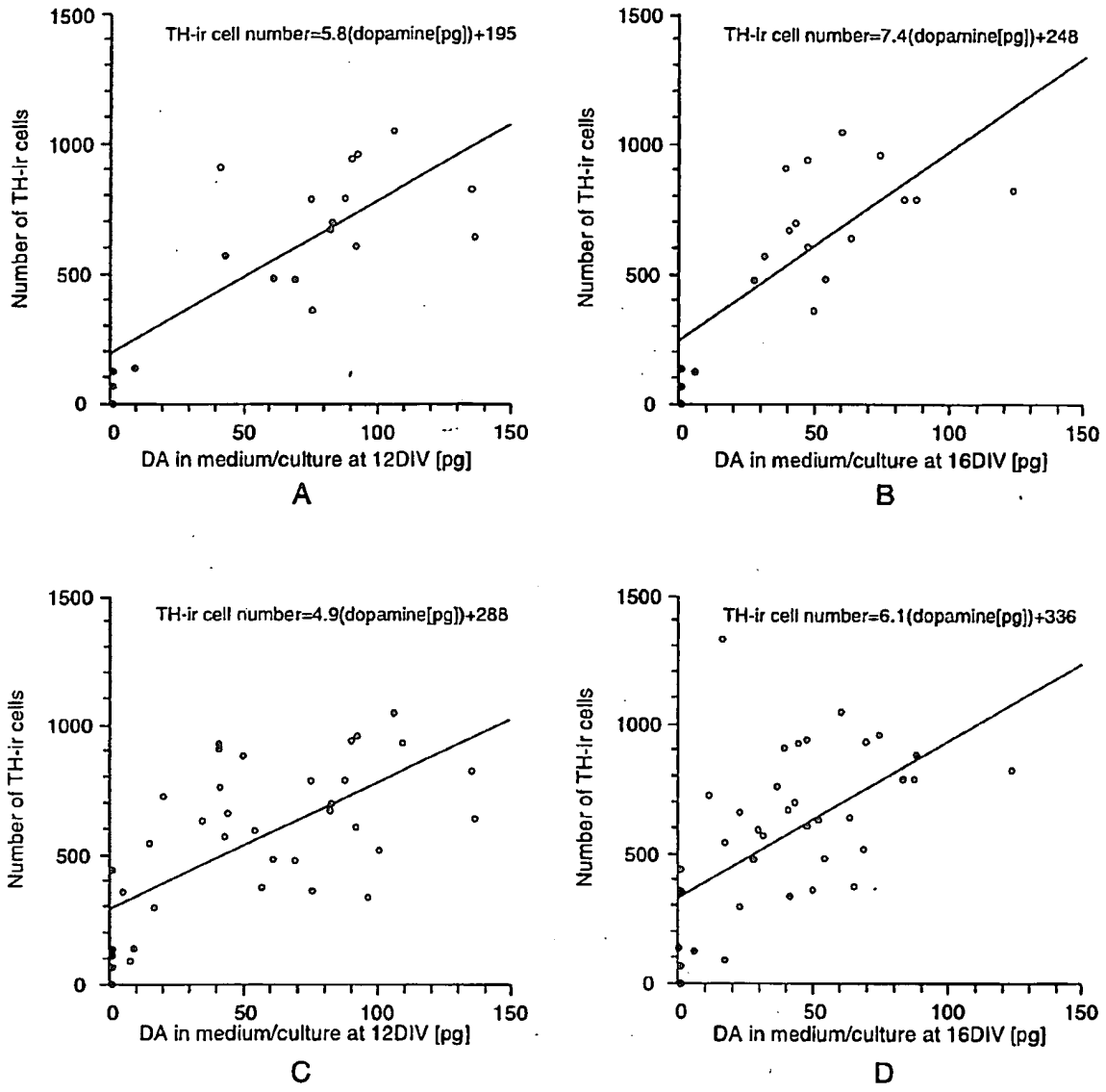

FIG. 5. Correlation between dopamine content and number of TH-ir cells for rostrally derived cultures at 12 DIV (A) and 16 DIV (B). Correlation between dopamine content and TH-ir cell number pooled for rostrally and caudally derived cultures at 12 DIV (C) and 16 DIV (D). The functions of the linear fits are given on the top of each figure. A linear relationship between dopamine content and TH-ir cell number is assumed for cultures with more than 250–300 TH-ir neurons. Cultures with fewer TH-ir cells are without dopamine or with very low dopamine levels.

tioned medium derived from rat fetal mesencephalic cultures. The relatively low dopamine level seen in our study (52 ± 7 pg/ml at 12 DIV) could be attributed to the very small culture volume [1 culture (0.27 mg–1.9 mg) per 1 ml medium] and the lack of KCl substitution and dopamine stabilizing agents in our medium. Dopamine levels in the medium as extrapolated for a culture size of 1 g would correspond to 20–200 ng in our study.

Dopamine Levels in the Culture Medium

A significant variation in dopamine levels over time could only be observed in rostrally derived cultures. Important factors that may influence dopamine levels in the medium are the TH-ir cells number in a culture at day zero, the survival rate of the neurons during culturing, and the dopamine production rate and reuptake capacity of individual neurons. Highest dopamine levels in the medium of rostrally derived cultures were detected at 12 DIV, which corresponds to chronological age postnatal day 4 (P4) for E13 tissue. The increase in DA levels between 5 and 12 DIV could result from differentiation of the dopaminergic neurons in the cultures. This is supported by the general observation of neuritic sprouting in this culture model [34]. A decrease in dopamine levels at 16 DIV may reflect a cell loss [9,36]. Significant differences between rostrally and caudally derived cul-

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tures at 12 DIV might be explained by the fact that TH-ir neurons first appear in the rostral mesencephalon at E12.5 [32]. Fractionated explantation at E13, when most of the future dopaminergic neurons are born according to autoradiographic studies [2,23,27] should lead to a higher yield of nigral dopaminergic neurons in rostral rather than in caudal portions. However, further work is needed to study local dopamine gradients at various developmental stages and additional sites in the mesencephalon to assess the relative effects of cell number, migration [27], phenotype induction and differentiation of nigral dopaminergic neurons and neuroepithelial progenitor cells on dopamine levels.

Correlations Between Dopamine Levels at Different Times In Vitro

Comparing dopamine levels of individual cultures at different time points a higher variability between 4 and 8 DIV than between 12 and 16 DIV was seen as evidenced by the lower correlation coefficients. This is due to the fact that the phase shortly after explantation is critical for cultures. Some cells committed to a dopaminergic phenotype need a certain period to start dopaminergic differentiation and other neurons, which are well differentiated at explantation, may be more vulnerable to physical damage and die after a few days in vitro. Thus, some cultures may switch on and others switch off dopamine production in vitro.

Correlation Between TH-ir Cells and Dopamine Content

Development of cells committed to a dopaminergic phenotype has been extensively described for the rat mesencephalon ([28,32,38], for review [21]). It has been estimated that, in the adult rat substantia nigra, between 13,000 and 15,000 dopaminergic neurons are present on each side [18]. Dopamine levels derived from whole-brain measurements demonstrate a steep increase between E15 and P20 (for review [21]). Between P30 and P60 dopamine concentrations have been found to increase to adult levels. The present study does not rely on whole brain measurements but has measured dopamine levels in conditioned medium of mesencephalic parts obtained by a fractionated explantation technique. Therefore, no bias due to the differential developmental speed of mesencephalic and nonmesencephalic dopaminergic CNS regions interferes with the data. The similarity between the frequency histograms for dopamine levels in the medium and for TH-ir cell numbers (Fig. 4) is an indication that a direct linear relationship between these two parameters exists, though it remains unclear whether this correlation is limited to the in vitro time used in the present study or will be valid for other developmental stages, too. The linear mathematical model applied on the data (see Fig. 5) suggests that the average amount of dopamine measured on a per neuron basis was 68 ± 8 fg at 16 DIV. However, this approximation has to be interpreted with caution, because the linear fit was less satisfactory for cultures with very low dopamine levels (Fig. 5). Moreover, TH-immunoreactivity in the present study was a necessary but not sufficient condition for detectable dopamine levels in the medium, as dopamine was never detected in cultures without TH-ir cells but few cultures with a low number of TH-ir cells present remained below detectability.

The identification of fetal ventral mesencephalon in E13–E14 rats is a relatively standardized procedure [8]. Therefore the probability of explanting tissue without any dopaminergic neurons due to identification errors is small. Explantation of ventral mesencephalon from human fetal tissue is more difficult. A high variation in the number of dopaminergic neurons per culture has been observed [34], as some cultures are from the center of the anlage of the substantia nigra while others are from the border. Thus, it can be expected that dopamine measurements in the medium of human FFRT cultures could be useful in a clinical setting to exclude cultures not derived from the proper substantia nigra. However, further studies are needed to elucidate the role of TH-ir cell number on dopamine levels in human tissue.

In conclusion, preoperative determination of dopamine content in the medium of FFRT cultures of rat ventral fetal mesencephalon is feasible during routine medium change and values can be reliably correlated to the number of TH-ir neurons present in each culture.

Further studies will have to demonstrate the correlation between dopamine content of the culture medium and behavioral changes after the transplantation of cultures with high dopamine levels compared with cultures with low dopamine levels. Dopamine measurements may also be an efficient mean to define the potential of various neuroprotective and neurotrophic treatment regimes prior to transplantation. Finally, dopamine determinations in medium of human fetal mesencephalic cultures might aid to control for correct tissue identification, handling and selection of tissue rich in dopaminergic neurons in future clinical neural transplantation.

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